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09/775,517	02/02/2001	Gregory Grabowski		1629

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EXAMINER

WEBER, JON P

ART UNIT PAPER NUMBER

1651

DATE MAILED: 04/07/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Interview Summary

Application No.

09/775,517

Applicant(s)

GRABOWSKI ET AL.

Examiner

Jon P Weber, Ph.D.

Art Unit

1651

All participants (applicant, applicant's representative, PTO personnel):

(1) Jon P Weber, Ph.D.

(3) Greg Grabowski

(2) Karlyn Schnapp

(4) _____

Date of Interview: 05 April 2004.

Type: a) ☐ Telephonic b) ☐ Video Conference
c) ☒ Personal [copy given to: 1) ☐ applicant

2) ☒ applicant's representative]

Exhibit shown or demonstration conducted: d) ☒ Yes e) ☐ No.

If Yes, brief description: power point presentation.

Claim(s) discussed: 1.

Identification of prior art discussed: all.

Agreement with respect to the claims f) ☒ was reached. g) ☐ was not reached. h) ☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussion centered around confusion between HSL and LAL. Prior art cited is actually discussing HSL not LAL. Based on Escary, would expect that atherosclerosis increase with added enzyme. LAL unexpectedly decreases. Will present Declaration from Hui as well as additional arguments to further clarify. May amend claims to exogenous LAL.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.


Examiner's signature, if required

The Role of LAL

Common pathway to control of cholesterol
and triglyceride metabolism

LAL and HSLipase Are Different Enzymes

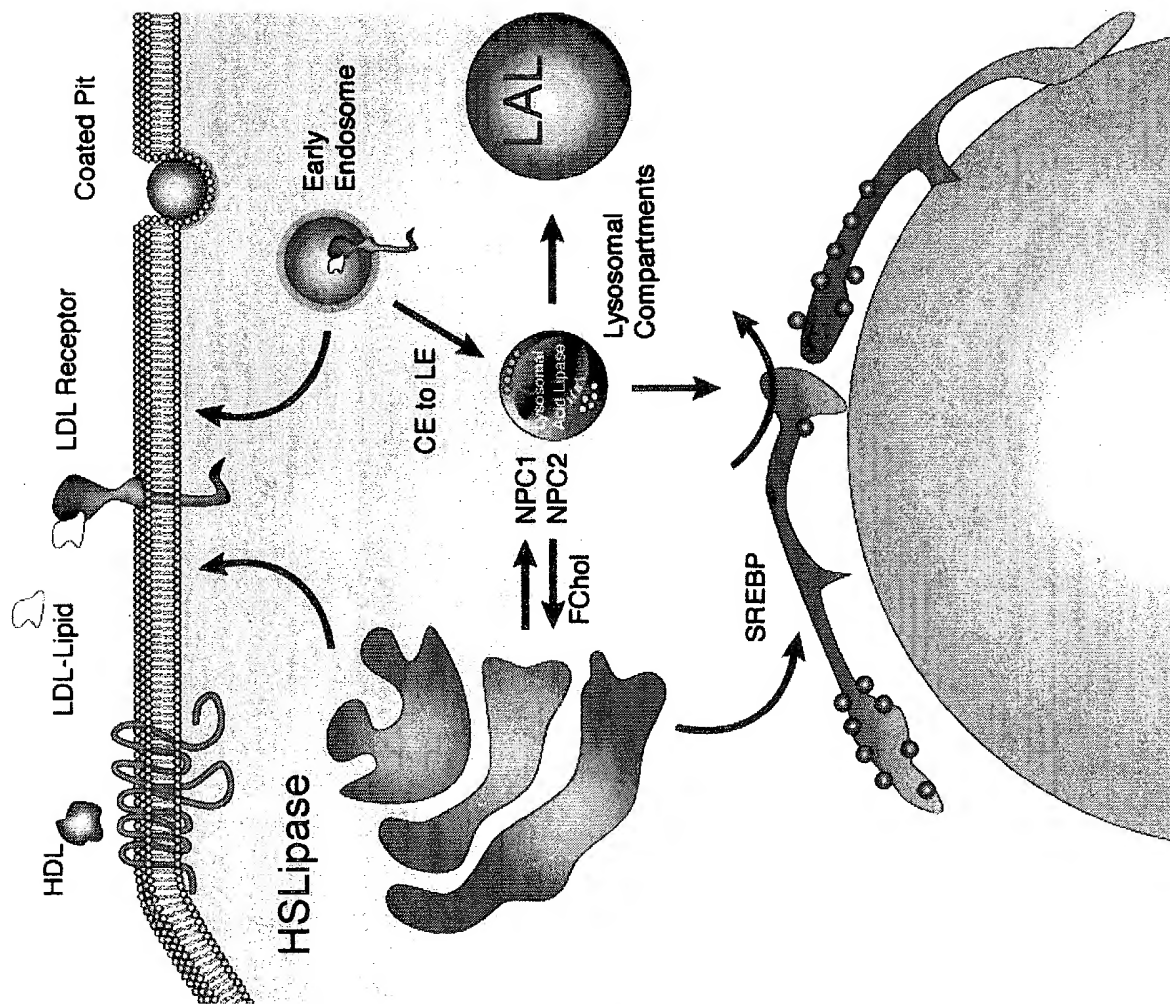
- Different cell compartments
 - LAL in lysosomes
 - HSLipase in cytoplasm
- Different amino acid sequences
- Different genes
- Different post-translational modifications
- Different developmental/tissue expression
- Different functions in cell

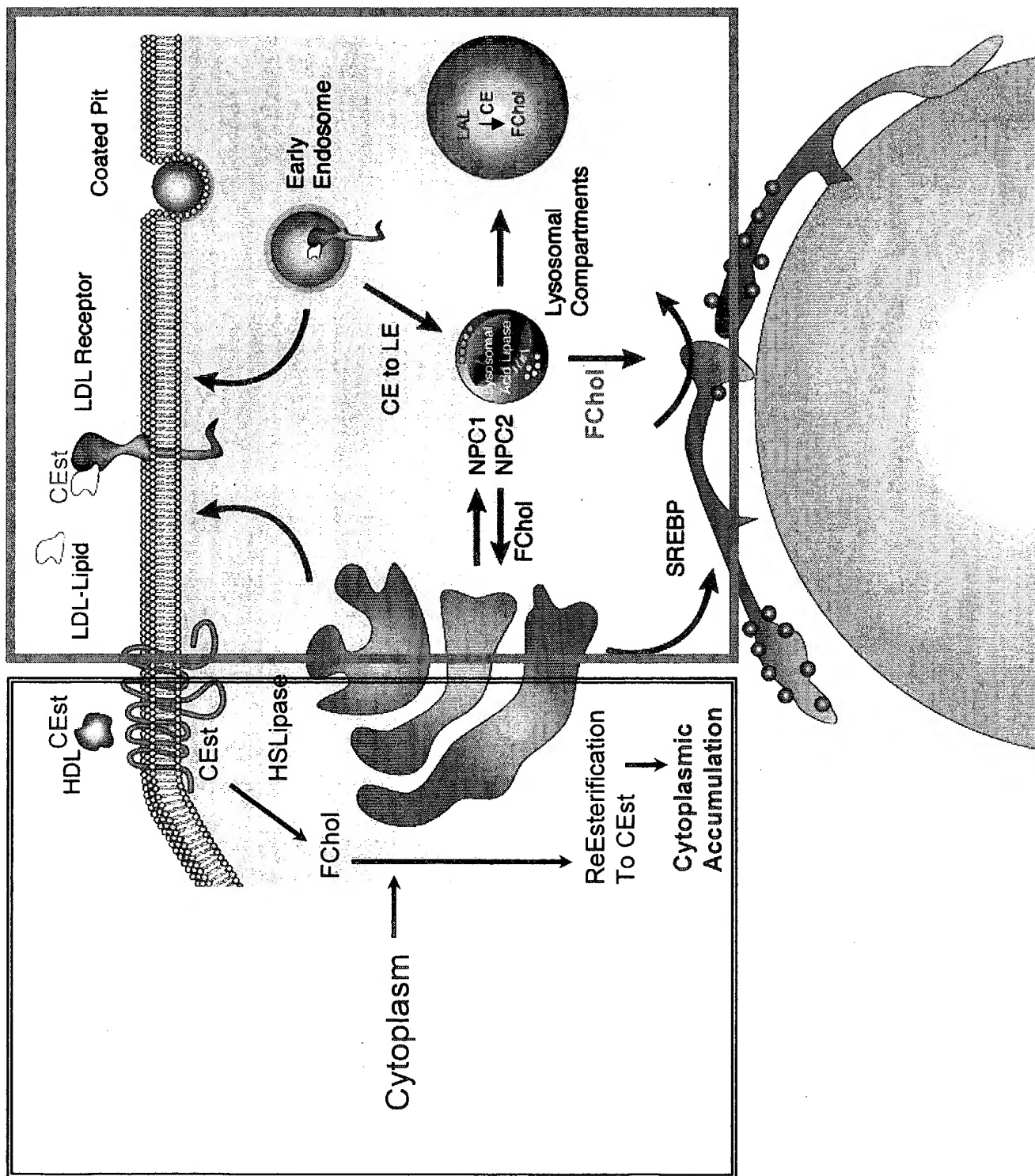
The Metabolic Role of LAL

Hydrolysis of cholesteryl esters and triglycerides delivered to Lysosome by the LDL receptor-mediated endocytotic pathway
Liberated free cholesterol and fatty acids signal many downstream events

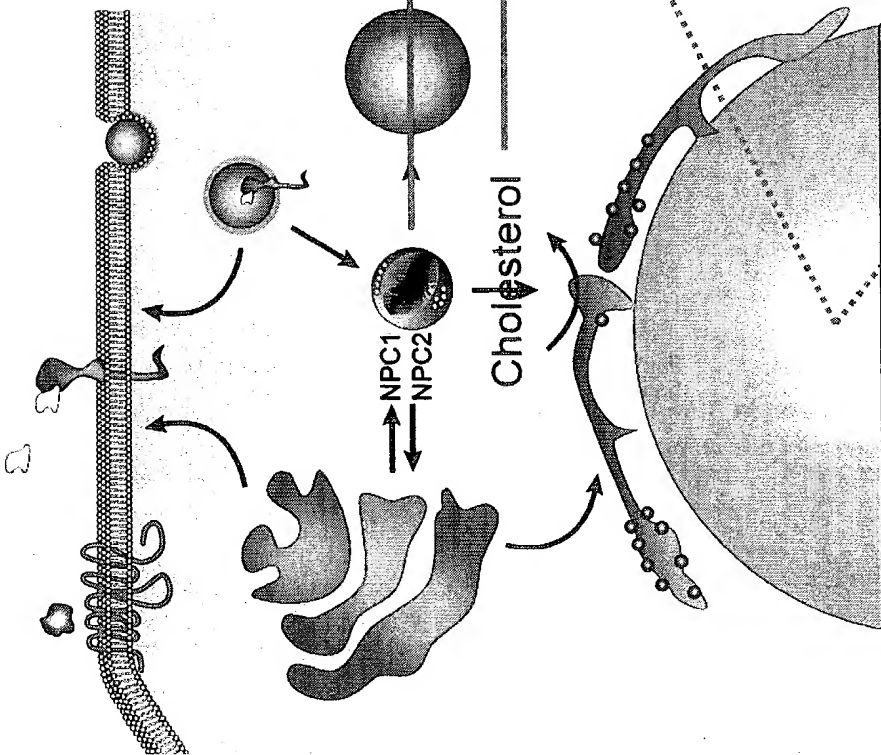
The Metabolic Role of Hormone Sensitive Lipase (HSLipase)

Hydrolysis of cholesteryl esters delivered to
the Cytoplasm by HDL mediated transfer
Liberated free cholesterol is re-esterified





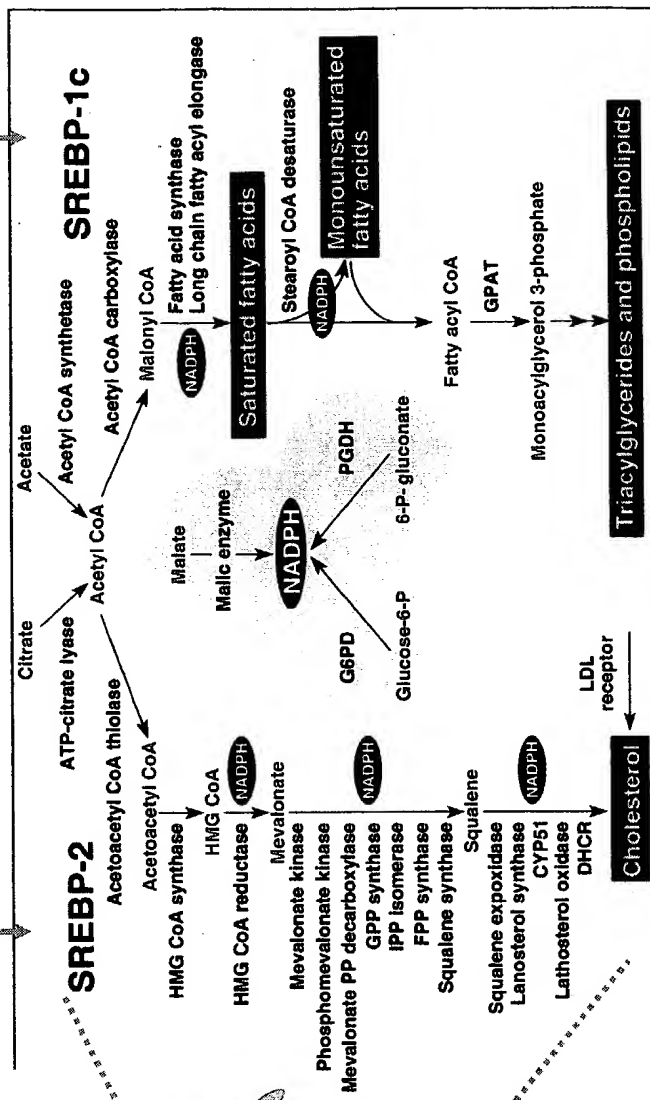
LDL-CE



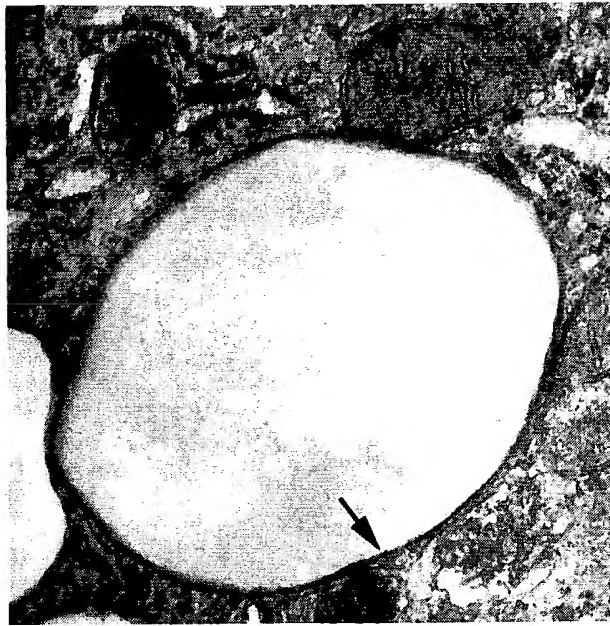
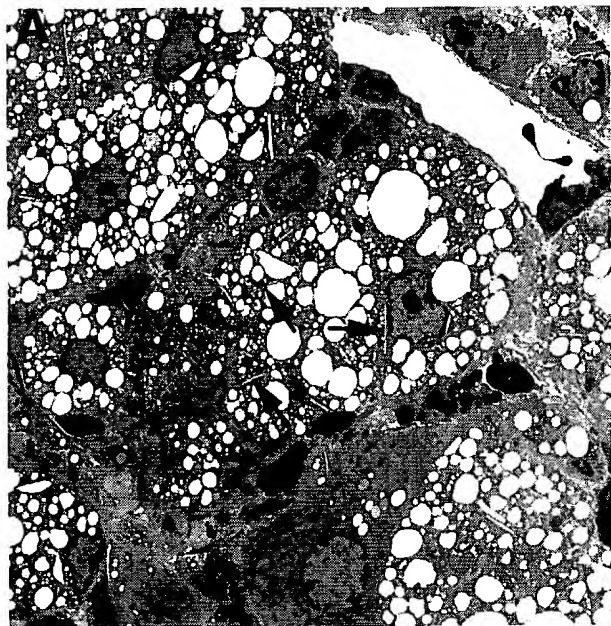
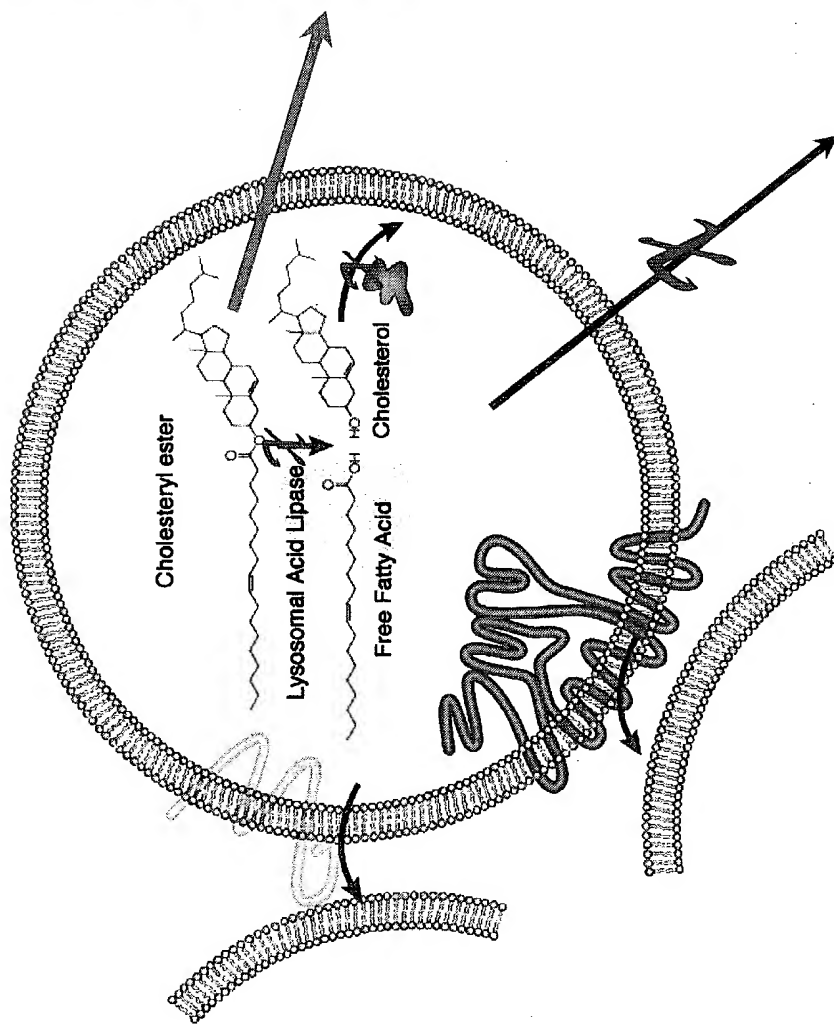
PPAR γ \longrightarrow Cytokines

Free Fatty Acids

Cholesterol



Lysosomal Acid Lipase Deficiency



HLAL 33	10	20	30	40	50	60
h-HSL 33	M K M E F L G L V V C L V L W P - - - L H S E G S G - - -	M D L E T M T Q S L V T L A E D N I A F F S S G G E T A	M R			
HLAL 33	40	50	60			
h-HSL 33	Q R L S G V F A G V R E Q A L G L E P A L G R L L G V A H L	Q R L S G V F A G V R E Q A L G L E P A L G R L L G V A	Q R L S G V F A G V R E Q A L G L E P A L G R L L G V A			
HLAL 33	70	80	90			
h-HSL 33	T A V D P P E T N M N V S E I I S Y W G F P S E E Y L V E T E	F D L D P P E T P A N G Y R S L V H T A R C C L A H L L H K S	F D L D P P E T N			
HLAL 33	100	110	120			
h-HSL 33	D G Y I L C L N E I P H G E K N H S D K G - - - - -	R - Y V A S S N R R R R I F F E R T S H H N L A E	R - Y V A S S N R R R R I F F E R T S H H N L A E			
HLAL 33	130	140	150			
h-HSL 33	Q L R A L V Y Y A Q R L L V T	Q L R A L V Y Y A Q R L L V T	Q L R A L V Y Y A Q R L L V T			
HLAL 33	160	170	180			
h-HSL 33	A D S S N W V T N L A N - - - S S L G F I L A D A G F D	A D F L R E Y V T L H K G C F Y G R C L G F Q F T P A	A D S S N W V T N L A N - - - S S L G F I L A D A G F D			

	190	200	210
HLAL 33	V W M G N	R K H K T	L S V S Q D
h-HSL 33	F L Q T I	S R N E T G	L S V A S S
	S I S I S	W T V K R R	S L S L
	S G L V S F G	R	L S V

	220	230	240
HLAL 83	- - - - -	- - - - -	- - - - -
H-HSL 83	F T S G R F A I D P E L R G A E F E R I T Q N L D V H F W K	- - - - -	- - - - -
	F T S G R F A I D P E L R G A E F E R I T Q N L D V H F W K	- - - - -	- - - - -

	250	260	270
HLAL 33	E	F	F
h-HSL 33	A	F	F

	2000	2000	2000	2000
HLAL 03	- - -	A F S S Y D E M	A K Y D L P	A S I N F I L N K T G P
H-HSL 03	P P E A F	P P E M P L T A	A D P T L T	H T G P G P
	P P E A F	A	L	L T G P G P

	710	.220	.220
HLAL 93	- - - - -	V Y Y V G H S Q G - - T	
H-HSL 93	V L V R L I S Y D L R E G Q D S E E	L S S L I K S N G Q R E S	
	V L V R L I S Y D L R E G Q D S E	.	S . G Q R .

HLAL 03
h-HZL 03

T I G F I A F S Q I P E L A K R I K M F F A L G P V A S V A 260
L E L W P R P Q Q A P R S R S L I V H F H G G G F V A Q T S 250
Q . P Q . P . . G V A 240

HLAL 33 270 F C T S P - - - - - 290 AK L GR
 h-HSL 33 R S H E P Y L K S V A Q E L G A P I I S I D Y S L A P E A P

HLAL 33 410 L P D H L I K D L F G D K E F L P Q S A F L K W L G T H V C
 h-HSL 33 F P R A L E E C F F A Y C W A I K H C A L L G S T G E R I C
 P L F .

HLAL 33 430 T H V I L K E L C G N L C F - - - - - 450
 h-HSL 33 - - L A G D S A G G N L C F I V A L R A A A Y G V R V P D G
 T H . . . G N L C F T V A L R A A A Y G V R V P D G

HLAL 33 460 - - - - - 480 L C G F N E R N
 h-HSL 33 I M A A Y P A T M L Q P A A S P S R L L S L M D P L L P L S
 I M A A Y P A T M L Q P A A S P S R L L S L

HLAL 33 490 L N M S R V D V Y T - - - - - 510
 h-HSL 33 V L S K C V S A Y A G A K T E D H S N S D Q K A L G M M G L
 V . Y G A K T E H S

HLAL 33 520 S Q A V K F Q K F Q A F D W G S S - - - - - 540
 h-HSL 33 V R R D T A L L R D F R L G A S S V L N S F L E L S G R K
 F G S S V L N S F L E L S G R K

550

560

550

HLAL 33

h-HSL 33

S Q K M S E P I A E P M R R S V
S Q K M S E P I A E P M R R S V

A K N Y F H Y N Q S Y P P T
S E A A L A Q P Q G P L G I T

600

590

590

HLAL 33

h-HSL 33

Y N V K D M L V P T A V W S G H D W L A D V
D S L K N L T L R D L S L R G N S E T S S D T P E M S L S A
K
P E M S L S A

620

620

610

HLAL 33

h-HSL 33

E T L S P S T P D V N I L L T
E T L S P S T P D V N I L L T

P E D A G E E A E A K N E L
P E D A G E E A E A K N E L

660

650

640

HLAL 33

h-HSL 33

S P M D R G L G V R A A F P E W E H L D
S P M D R G L G V R A A F P E W E H L D

R S S Q G A T Q M P
R S S Q G A T Q M P

690

690

670

HLAL 33

h-HSL 33

L Y S S P I V K N P F F I W G L D A P W R L Y N
L Y S S P I V K N P F F I W G L D A P W R L Y N

S L P P V H I
S L P P V H I

720

710

700

HLAL 33

h-HSL 33

V A C A L D P M L D D S V M L A E R R N L G Q P V T L R V
V A C A L D P M L D D S V M L A E R R N L G Q P V T L R V

HLAL 33

h-HSL 33

750

750

750

V E D L P H G F L T L A A L C R E T R Q A A E L C V E R I R
V E D L P H G F L T L A A L C R E T R Q A A E L C V E R I R

750

750

750

Reduction of Atherosclerotic Plaques by Lysosomal Acid Lipase Supplementation

Hong Du, Susan Schiavi, Nick Wan, Mark Levine, David P. Witte, Gregory A. Grabowski

Objective—Proof of principle is presented for targeted enzyme supplementation by using lysosomal acid lipase to decrease aortic and coronary wall lipid accumulation in a mouse model of atherosclerosis.

Methods and Results—Mice with LDL receptor deficiency were placed on an atherogenic diet and developed predictable aortic and coronary atheroma. α -Mannosyl-terminated human lysosomal acid lipase (phLAL) was produced in *Pichia pastoris*, purified, and administered intravenously to such mice with either early or late lesions. phLAL injections reduced plasma, hepatic, and splenic cholesteryl esters and triglycerides in affected mice. phLAL was detected in hepatic Kupffer cells and in atheromatous foam cells. Repeated enzyme injections were well tolerated, with no obvious adverse effects. In addition, the coronary and aortic atheromatous lesions were (1) eliminated in their early stages and (2) quantitatively and qualitatively reduced in their advanced stages.

Conclusion—These results support the potential utility of lysosomal acid lipase supplementation for the treatment of atherosclerosis, a leading cause of mortality and morbidity in Westernized nations. (*Arterioscler Thromb Vasc Biol.* 2004;24:147-154.)

Key Words: lysosomal acid lipase ■ atherosclerosis ■ lesion ■ mice

Atherosclerosis is the number 1 cause of mortality and morbidity in the developed countries. A number of interventions or preventions delay the consequences of atherosclerosis, eg, low cholesterol diet and exercise, HMG-CoA reductase inhibitors, and coronary artery bypass. However, they are not suitable for all patients, and few have been shown to promote regression of lesions. Therefore, new approaches are needed for the treatment and prevention of atherosclerosis. Several stages characterize the progression of atherosclerotic lesions.^{1,2} The earliest lesion is the "fatty streak," an aggregation of lipid-rich macrophages and T-lymphocytes within the intima layer of an artery. The fatty streaks evolve into intermediate lesions that have a layer of foamy macrophages and smooth muscle cells. This develops into complex and occlusive lesions, as well as fibrous plaques. These plaques have a dense cap of connective tissue, with embedded smooth muscle cells overlaying a core of lipid and necrotic debris. Macrophages are present at all lesional stages with excessive cholesterol and cholesteryl esters in lysosomes. Continuing development of atherosclerotic plaques requires progressive macrophage processing of cholesteryl esters in and through the lysosomes. Perpetuation and maturation of the plaques depends on additional complex inflammatory and scarring processes. Lysosomal acid lipase (LAL) is the only hydrolase for cleavage of cholesteryl esters delivered to the lysosomes.³

The receptors that mediate cholesteryl ester uptake into macrophage include those for LDL and oxidized LDL (oxLDL), ie, LDL receptor (LDL-R), the scavenger receptors type AI and AII (SR-AI and SR-AII),⁴⁻⁶ the scavenger receptor type B1 (SR-B1),⁷⁻⁹ CD36,¹⁰ and the LDL receptor related protein (LRP).¹¹ All of these, except SR-B1, direct associated lipids to the lysosome.¹²⁻¹⁵ Furthermore, modification of LDL by oxidation, aggregation, and glycation occurs in the atherosclerotic lesions by histochemical and biochemical studies.¹⁶⁻²⁰ The delivery of oxidized LDL to lysosomes of macrophages decreases cholesteryl ester hydrolysis, apolipoprotein degradation, and cholesterol to acyl-CoA/cholesterol acyltransferase (ACAT) activity.²¹⁻²⁵ Aggregated LDL induces cholesteryl ester accumulation in cultured macrophages.^{18,20,26,27} Also, LDL particles, modified by advanced glycation, promote cholesterol accumulation in cultured macrophages.^{19,28} Consequently, enhancement of LAL activity in macrophages could provide a means to decrease accumulated, pathologic cholesteryl esters and triglycerides (TGs) that are causally related to atherosclerosis.

LAL is an essential enzyme for the cleavage of cholesteryl esters and TGs delivered to the lysosomes. The biochemical phenotype of the LAL knockout mouse indicates that other lipases cannot compensate for the loss of LAL activity in the lysosomes.³ The endogenously synthesized mature, soluble

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glycoprotein has 372 amino acids, is trafficked to the lysosome by the mannose-6-phosphate receptor, and has active site properties similar to those of other lipases.^{29,30} LAL contributes to cholesterol and fatty acid homeostasis by several mechanisms. Once cholesteryl esters are cleaved by LAL, free cholesterol exits the lysosome and participates in the modulation of cholesterol and fatty acid metabolism via the sterol regulatory element-binding protein (SREBP) system.^{31–34} Free cholesterol, a product of LAL hydrolysis of CE, leaves the lysosome and leads to downregulation of endogenous cellular cholesterol synthesis and LDL receptor-mediated uptake through the cholesterol sensing mechanisms of the cell. The free fatty acids generated by LAL and their metabolites released from the lysosome could be ligands for the peroxisome proliferated-activator receptor- γ (PPAR γ), a master regulator of lipogenesis, macrophage maturation, anti-inflammation, and glucose homeostasis in the body.^{13,35–38} Deficiency of LAL via targeted gene disruption leads to a multisystemic disease that includes the phenotype of human Wolman disease, aberrant cholesterol and TG metabolism, and macrophage proliferation.³⁹ These diverse effects support the important and central role of LAL in the control of cholesterol and fat metabolism and macrophage proliferation, as well as a potential role in the treatment of atherosclerosis.

Expression of the human LAL (hLAL) in *Pichia pastoris* (phLAL) systems produced active hLAL that was targeted to macrophage lysosomes.³⁹ Intravenous injections of phLAL into LAL-deficient mice (*lal*^{-/-}) corrected the lipid storage phenotype in multiple tissues.³⁹ Here, phLAL was administered to the mouse model of atherosclerosis, the LDL-receptor knockout (*ldlr*^{-/-}) mice on a high fat/high cholesterol diet (HFCD). Repeated doses of phLAL resulted in almost complete elimination of early stage lesions and significant improvement in the quality and quantity of advanced lesions.

Methods

Animal Models

Homozygous *ldlr*^{-/-} and *apoE*^{-/-} mice in a C57BL/6 background were from The Jackson Laboratory (Bar Harbor, Maine). Homozygous *lal*^{-/-} mice were created in this laboratory.³ Cross breeding of *lal*^{-/-} and *apoE*^{-/-} mice generated double knockout of *lal*^{-/-}; *apoE*^{-/-} mice. Mice were genotyped by PCR using tail DNA.³ The *ldlr*^{-/-} mice were fed a high fat/high cholesterol diet (HFCD, 7.5% cocoa butter fat, 1.25% cholesterol, TD86257, Harlan Tekland) from the age of 1.5 months. All animals had ad libitum access to food and water. The animal experiments were performed according to National Institutes of Health guidelines and were approved by IACUC at Cincinnati Children's Hospital Research Foundation. phLAL was produced in *Pichia pastoris* as previously described.³⁹

Study Design

Experiments were designed to evaluate the efficacy of LAL enzyme supplementation therapy to treat the athero/arterial-sclerosis at the early foam cell stage (group A) and at the advanced lesion stages (group B). The group A mice received phLAL (1.5 U/injection, n=8) after 1 month of HFCD, ie, beginning at 2.5 months of age. The group B mice received phLAL (6 U/injection, n=7) after 2 months of HFCD, ie, beginning at 3.5 months of age. The control mice for each group were injected with PBS (n=4 to 5). Mice in both groups started HFCD at 1.5 months of age and continued HFCD for the entire study period. PBS or phLAL injections (100 μ L) were done every third day for 10 injections. Mice were harvested 2 days after

the 10th injection. Tissues were processed for histologic, immunohistochemical, and biochemical analyses. phLAL was \approx 96% pure on silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Plasma Lipids, Lipoprotein Profile, and Tissue Lipids

Blood was collected from the inferior vena cava (IVC) of anesthetized mice (ketamine, acepromazine, and xylazine, 0.2 mL, intraperitoneal injection) after an overnight fast. Plasma was collected (5000g, 10 minutes, 4°C) and stored (4°C and -20°C). Plasma free cholesterol, cholesteryl esters, and TG concentrations were tested by using colorimetric assays (COD-PAP kit for free cholesterol, Wako Chemicals GmbH; TGs/GB kit and cholesterol/HP kit were from Boehringer, GmbH). Plasma from group B mice was pooled (200 μ L) and subjected to fast protein liquid chromatography by using 2 Superose 6 HR columns (Pharmacia Biotech Inc.).^{39,40} Cholesterol concentrations were determined in each fraction (0.5 mL; cholesterol/HP kits). Total lipids were extracted from liver and spleen by the Folch method.^{3,41} TG concentrations were estimated as described.^{39,42,43} Total tissue cholesterol concentrations were estimated using O-phthalaldehyde.^{39,43,44}

Tissue Preparation and Morphometric Analyses of Atherosclerotic Lesions

After blood collection under anesthesia, livers, spleens, and small intestines were removed and processed for histologic and lipid analyses. The hearts were perfused through the left ventricle with PBS followed by 10 mL of 4% paraformaldehyde. The aortas were isolated under microscopy and photographed. For qualitative analysis, hearts were split in half along the aortas, embedded in paraffin with the open aorta upward, and sectioned at 4- μ m thickness from midsagittal to parasagittal regions of the heart. Aortic arches were cut to 5 fragments at same relative positions, embedded in paraffin with distal end upward, and cross-sectioned at 4- μ m thickness from the distal end of each segment of the aorta. Samples were mounted on slides and stained with hematoxylin/eosin (H&E), elastic Van Gieson, or Gomori's trichrome. For quantitative analyses, 40 to 60 sections (7 μ m) were collected from each heart with same orientation and stained with H&E. Lesional area was defined by vessel wall involvement on H&E staining. Images of every fifth sections were captured with a digital camera and analyzed by blinded readers by using computerized morphometric (MetaMorph) software. Briefly, images at the same magnification were captured, and a grid (19 \times 19 lines) divided the image into 400 U. The area covered by lesional macrophages, collagen positive extracellular matrix and necrotic core, and the vessel wall were numbered and manually counted. The areas were converted into μ M² by using an integrated size marker in the MetaMorph program. Staining by Gomori's trichrome and elastic Van Gieson were used to identify the collagen positive areas, macrophage cell areas, and elastic vessel wall areas. The lesional area was defined from the first elastin positive vessel wall section. Extensive sections and H&E staining (160 to 210 sections, 7 μ m) were also performed for one representative heart in control group and 2 representative heart in treated group A. The positive coronary arterial lesions were scored by a blinded reader. The severity of the coronary lesion was scored by a percentage of the sections that had atherosclerotic lesions in arterial vessels and by an estimation of vessel area that was blocked by the lesion as defined in the legend for Table I (available online at <http://atvb.ahajournals.org>).

Immunohistochemical Staining

Immunohistochemical analyses were performed with frozen or paraffin-embedded aortic valve sections by using rabbit anti-hLAL antibody (1:500) or rat anti-mouse Mac-3 monoclonal antibody (PharMingen). Biotinylated conjugated anti-rabbit or anti-rat IgG (Vector) were used as secondary antibody, respectively.⁴⁵ The signal was detected by using VECTASTAIN ABC kit (Vector) and counter-stained with Nuclear Fast Red.

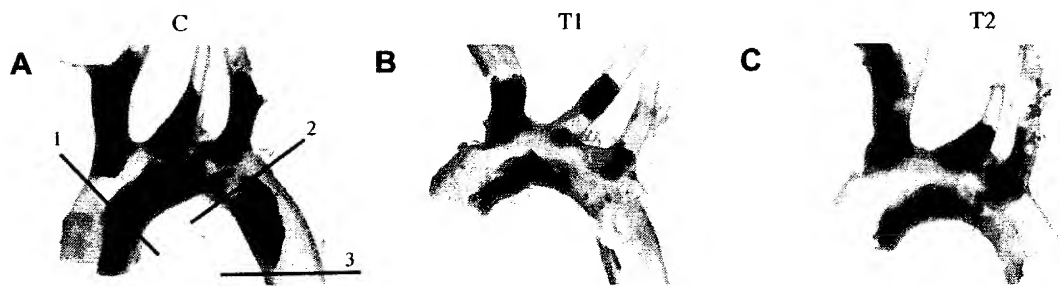


Figure 1. Effect of phLAL on aortic lesions: Representative aortic arches were isolated from typical controls after 10 injections of PBS (A) and 2 group B mice, T1 (B) and T2 (C), that are typical of this group after receiving 10 injections of phLAL. These were then photographed under transillumination (bright field). The dark lesions in the arch and the branch points of the brachiocephalic trunk are atherosclerotic plaques. Magnification, 25 \times .

Statistical Analysis

All data are expressed as mean \pm SEM. A 2-tailed unpaired *t* test was used to compare tissue lipid concentration, lesion area between PBS and phLAL treated mice. A $P < 0.05$ was considered significant.

Results

Reduction of Plasma and Tissue Lipids by phLAL

Two groups were studied: group A received a lower dose (1.5 U phLAL/injection, 60 U/kg), beginning at 2.5 months, and group B received higher doses (6 U phLAL/injection, 240 U/kg), beginning at 3.5 months. phLAL was given every third day for a total of 10 injections as tail vein boluses. Age-matched control mice received PBS injections. Both groups and their controls received HFCD beginning at 1.5 months, and the diet was continued throughout the study. Compared with wild-type mice, the plasma free cholesterol and cholesteryl esters were increased 9.1-fold and 4.7-fold, respectively, in *ldlr*^{-/-} mice on HFCD (Table I). In group A, phLAL reduced plasma free cholesterol (29.8%, $P = 0.089$) and cholesteryl esters (52.1%, $P = 0.0025$) relative to control mice. In group B, phLAL decreased plasma free cholesterol (27.6%, $P = 0.0135$), cholesteryl esters (28.6%, $P = 0.0107$), and plasma TGs (46.2%, $P = 0.0045$) in *ldlr*^{-/-} mice compared with their controls.

In control *ldlr*^{-/-} mice receiving HFCD, cholesterol and (TGs) levels in liver and spleen were increased relative to those of wild-type mice receiving chow diet (Table I). Decreases in total hepatic (43.2%, $P = 0.002$) or splenic (52.6%, $P = 0.0351$) cholesteryl esters were observed in group B mice. Total hepatic TGs were reduced by 34.9% and 62.2% in groups A and B, respectively ($P = 0.002$ and $P = 0.0218$). Total splenic TGs were reduced by 46.6% and 70% in groups A and B, respectively ($P = 0.0183$ and $P = 0.0589$).

Effects of phLAL Injections on Lipoprotein Profiles

The plasma lipoprotein profiles were compared between control and phLAL treated mice in group B. A large amount of VLDL cholesterol accumulated in *ldlr*^{-/-} mice receiving HFCD for 3 months. Treatment with phLAL did not alter the VLDL cholesterol, but there was a small consistent decrease in IDL/LDL cholesterol in lipoprotein profile.

Reduction in Atherosclerotic Lesions in the Aorta and Aortic Valves

To evaluate the gross effects of phLAL injections on the progression of atherosclerotic lesions, whole mounts of aortic arches were examined by transillumination. In group A, all (3/3) control mice showed extensive lesions of the arch and branch points for the major vessels, eg, brachiocephalic arteries. The lesions were particularly prominent at the lesser curvature of the arch and the subclavian branch points. Treatment of phLAL at lower dose (1.5 U/injection) had little effect on the extent of the lesions in aorta of *ldlr*^{-/-} mice (data not shown).

With high dose-treated mice (group B), the lesional area was reduced in the lesser curvature of aortic arch. The lesions at the bifurcation points of major vessels were similar in the test and control mice (Figure 1). Lesional reductions in isolated aortas from 5 enzyme treated mice were somewhat variable (Figure 1B and 1C), but this variation did not affect the significance of lesional reduction by phLAL treatment.

Histologic analyses of atherosclerotic lesions of group A mice are in Figure 2 (typical results). Five of 8 group A mice were processed for aortic valve foam cell accumulations and

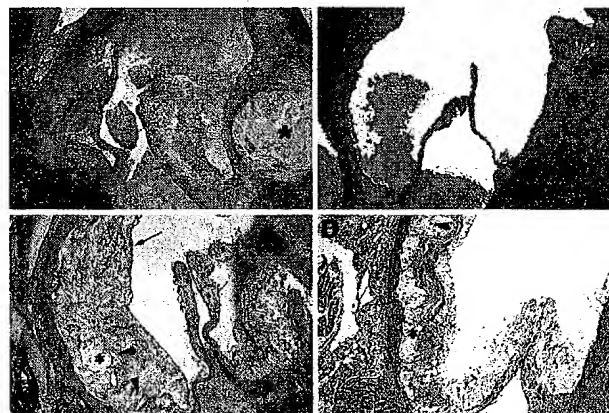


Figure 2. Histology of typical groups A and B and control mice. Representative H&E (100 \times) aortic valve sections of group A mice—control (A) and phLAL-treated (B)—and group B mice—control (C) and phLAL-treated (D). The asterisks indicate necrotic zones next to disrupted medial layers. The arrow indicates a fibrous cap, and arrowheads highlight cholesterol crystals. Reduced foamy cells were evident in the lesions of the aortic valve lesions in group A (B) or in group B (D) mice.

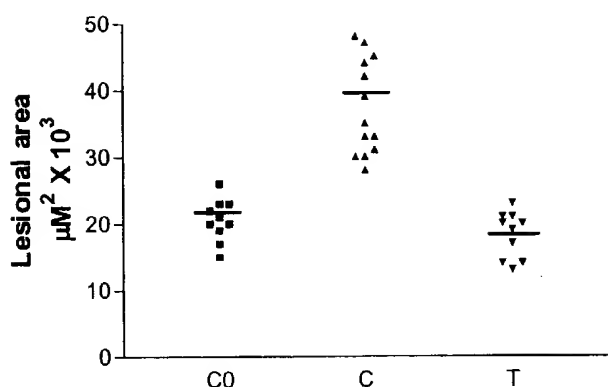


Figure 3. Quantitative analyses of aortic valve lesional areas. Lesion areas of *ldlr*^{-/-} mice on HFCD before receiving (C0) either PBS (C) or phLAL (T) injections. The lesion area was defined from the first elastin positive area. C and T mice each received 10 injections of PBS or phLAL (6 U/injection) and were analyzed 2 days after injection 10. *n*=5 for each group, *P*=0.0115 for C vs T.

these showed major reductions [from ++++ (30% to 40% of aortic valve areas, AVAs), to ++ (10% to 19% of AVA); 2/5, or 0 (<10% of AVA, 2/5)] (Figure 2B). In control mice the aortic valves showed large atherosclerotic lesions with large numbers of foam cells (Figure 2A).

In control mice for group B, the atherosclerotic lesions at 4.5 months were very complex with fibrous caps, necrotic cores, and cholesterol crystals (Figure 2C). In comparison, phLAL-treated mice showed reductions, but not elimination, of atherosclerotic lesions in the aortic valve (Figure 2D). The number of foam cells was greatly decreased, but the necrotic core and cholesterol crystals remained. Quantitative analyses of the lesional areas were conducted by using serial sections of the aortic valve from the control and group B (*n*=5 in each). Every fifth section (10 μm) was mounted and stained for a total of 15 sections from each heart. This covered ≈750 μm around the aortic valves. The lesional areas on the aortic valves were quantified by using computerized morphometric software (MetaMorph). Significant reductions (*P*=0.0115) in lesional area were found in phLAL-treated mice compared with controls. The mean lesional area in phLAL-treated mice was similar to the pretreatment values (Figure 3).

Effects of phLAL Treatment on Coronary Artery Lesions

Control mice for groups A and B had extensive multifocal lesions in the coronary arteries. All had heavy infiltration with foamy macrophages and plaques extending a considerable distance into the coronary arteries. In one case, the main branch of the left coronary artery was completely obliterated with an advanced lesion containing cholesterol crystals and inflammatory cells (Figure 1, available online at <http://atvb.ahajournals.org>). In comparison, 7/8 of the phLAL treated mice in group A had normal coronary vessels (Figure 1). One enzyme treated mouse in group A had foamy cells in a small intramuscular coronary vessel. The other coronary arteries in this mouse did not have lesions. These results were

confirmed in a more quantitative assessment of the coronary arterial lesions as obtained by sequential H & E sections (total=210 sections; 10 μm thickness) of the hearts from a control and 2 treated group A mice. The control mouse had multiple severe plaques (filled 80% to 100% area of coronary artery lumen) in the coronary arteries in 48% of the sections. One treated mouse had completely normal coronary arteries, and another treated mouse had mild lesions (filled 7% to 24% area of artery lumen) in the coronary arteries in 10% of the sections.

Reduction in the Complexity of the Lesions in phLAL-Treated *ldlr*^{-/-} Mice

The effects of phLAL on the complexity of atherosclerotic lesions in group B mice were evaluated by histological analyses of lesional elastin and collagen staining. The aortas from group B control and treated mice were sectioned into 5 fragments (3/5 segment positions) (Figure 1A). Sections from distal end of fragment 1 were stained for elastin and collagen. Compared with the control mice, the group B treated mice had the lesional areas that were much smaller and with less extracellular matrix content, ie, collagen, and less vessel wall dilation (Figure 2, available online at <http://atvb.ahajournals.org>). The lesional areas and collagen contents in group B mice were similar to those isolated from the mice before the initiation of enzyme administration. The macrophage area and collagen positive area were quantitatively analyzed by MetaMorph software (Figure 2IG). The phLAL-treated mice had less area of macrophages and collagen-rich extracellular matrix. The atherosclerotic lesions from control mice were highly complex, with fibrous caps (Figure 4A and 4B, arrow), and large amounts of collagen (Figure 4D and 4E, blue). The clear areas within these advanced, complicated lesions had lipid or/and cholesterol crystal accumulation and necrotic cores (Figure 4A, 4B, 4D, and 4E, asterisk). Collagen-rich fibrosis was present in aortic valves of control mice (Figure 4B and 4E, arrowhead). In the group B treated mice the lesions were less complex, with lesser degrees of macrophage infiltration and fibrous caps were lacking (Figure 4C and 4F). The deposition of excess extracellular matrix also was much less in treated mice (Figure 4E versus 4F). The macrophage area, collagen positive area, and necrotic core were quantitatively analyzed by using MetaMorph software (Figure 5).

Macrophage Targeting of phLAL to the Atherosclerotic Lesions

Endogenous LAL protein in *ldlr*^{-/-} mice could confound these analyses. Thus, *ldlr*^{-/-}; *lal*^{-/-} mice were developed and placed on a HFCD. However, *lal*^{-/-}; *ldlr*^{-/-} mice died within a few days of HFCD initiation (data not shown). The alternative of *apoE*^{-/-}; *lal*^{-/-} mice was developed and used. These mice had no detectible LAL protein and developed spontaneous atherosclerotic lesions without dietary challenge (Figure 6A). By immunohistochemical staining, phLAL protein was taken up into the foam cells of atherosclerotic lesions (Figure 6C). Serial sections were stained with the macrophage marker, Mac-3 (Figure 6D), or oil red O (Figure 6B).

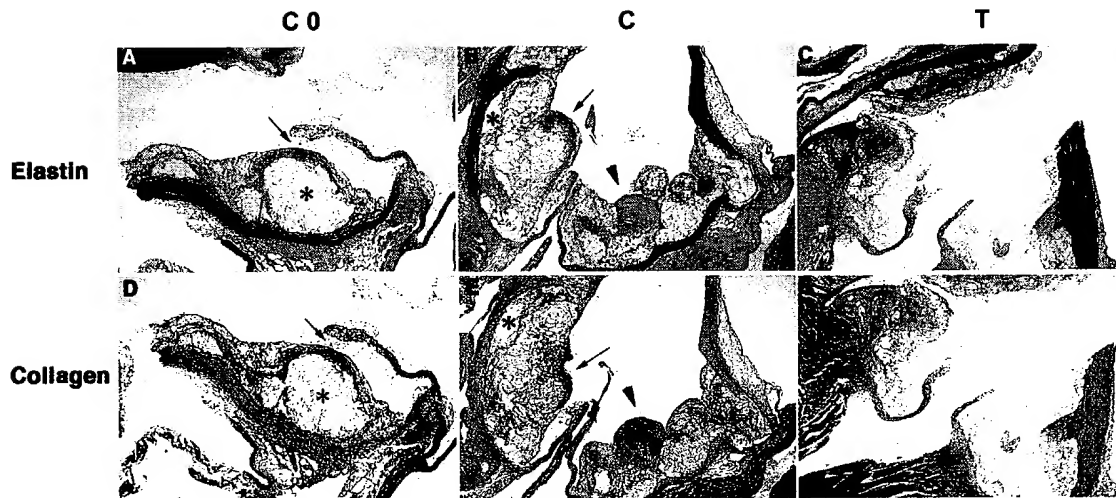


Figure 4. Reduced the foam cells in atherosclerotic lesion in aortic valve of group B treated mice. Sections of aortic valvular lesions were stained for elastin (A through C, black) and collagen (D through F, blue). C0, *ldlr*^{-/-} mice prior to injection at age 3.5 months. C, PBS (10 injections) control mice. T indicates phLAL-treated (10 injections) mice. *Area of necrotic core. Fibrous caps are indicated with arrow, and thrombosis is indicated with arrowhead. Notice the reduction the number of foam macrophages in phLAL treated samples. Magnification, 100 \times .

The results show uptake of phLAL protein into foamy macrophages and surface endothelial cells.

Survival and Antibody Development

All *ldlr*^{-/-} mice in group A survived. Fifteen of 17 *ldlr*^{-/-} mice in group B survived. In group B, 1 mouse died after 2 injections and another after 5 injections of phLAL. There was no obvious cause for the death. No other adverse effects were noted in either treated or untreated groups. All *ldlr*^{-/-} mice injected with phLAL developed anti-phLAL antibodies as assessed by Western blot analyses. One developed a high titer (1:32 000) antibody (data not shown). The earliest development of anti-phLAL antibody was after 5 injections (15 days). These antibodies were not inhibitory toward activity. The antibodies were directed against the LAL protein because they reacted similarly with the deglycosylated and the glycosylated proteins.

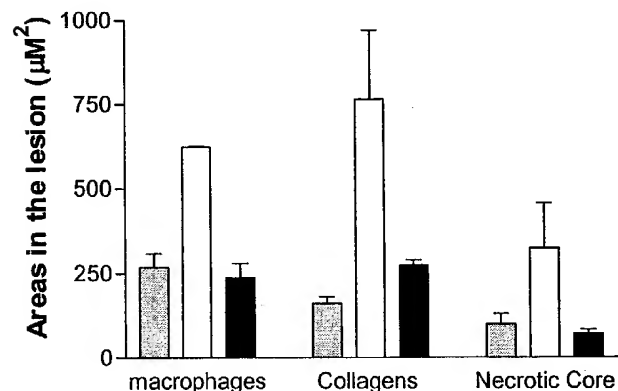


Figure 5. Quantitative analysis of areas in the lesion of aortic valve. The methods are detailed in *Methods*. The macrophage and collagen positive areas were defined by van Gieson and Gomori's trichrome stains.

Discussion

This study demonstrates that enzyme supplementation with phLAL had significant effects on the atherosclerotic lesions in *ldlr*^{-/-} mice receiving HFCD. The lesions were diminished or absent in treated mice compared with the extensive and very severe lesions in the untreated cohorts. This was most evident during early lesional development. For preexisting advanced lesions, treatment of LAL reduced the lesional area and the macrophage component and, thus, appeared to prevent progression of and stabilized the preexisting lesions. The majority of the enzyme was localized to the liver macrophages, Kupffer cells, by immunohistochemical staining after intravenous injection.³⁹ Biological activity of phLAL was

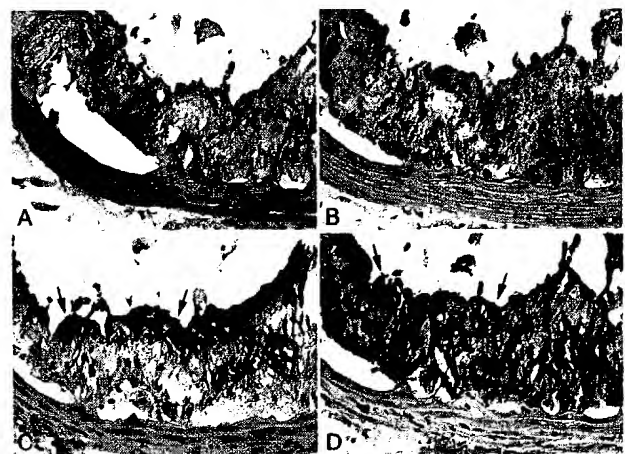


Figure 6. Localization of phLAL in foam cells of atherosclerotic lesion. Sections of the lesions at the aortic lesions of *ldl*^{-/-}; *apoE*^{-/-} mice stained with H&E (A), oil red O (B, red droplets indicates lipids), anti-phLAL antibody (C, brown), or macrophage marker, anti-Mac-3 (D, brown). The tissues were harvested 4 hours after injection of phLAL (24 U). LAL-positive cells were positive for Mac-3 (arrows). Not all macrophages have uptake of hLAL enzyme. Magnification, 400 \times .

assessed previously in *lal*^{-/-} mice.³⁹ Such in vivo activity also was evident from the reductions in tissue CEs and TGs in *lal*^{-/-} and HFCD-fed *ldlr*^{-/-} mice. Injection of pH LAL in the *ldlr*^{-/-} mice also led to significant reductions of plasma CE in group A mice, and of plasma free cholesterol, CE, and TG in group B mice. This suggested the dose-dependent systemic effect of pH LAL. The reduction of plasma lipids by pH LAL might be mediated by a decrease of lipoprotein production or an increase of lipoprotein uptake in these *ldlr*^{-/-} mice. Based on an effect of pH LAL in reduction of tissue lipid in the liver, we speculate that plasma lipid reductions are mediated though a decrease of lipoprotein production in these *ldlr*^{-/-} mice.

At least 3 mechanisms could account for the lesional reduction by pH LAL administration. First, LAL enters the lesional foam cells and hydrolyzes the stored CEs and TGs.⁴⁶ Electron microscopic analyses of lesions confirmed that much of the accumulated lipid in foam cells occurred within large, lipid-filled lysosome.⁴⁷ Most of the cholesterol in atherosclerotic lesions is esterified with linoleate rather than oleate,^{48,49} which indicates a LDL source rather than a product of ACAT.⁵⁰ Here macrophages in the atherosclerotic lesions of *apoE*^{-/-}; *lal*^{-/-} mice were positive for injected pH LAL. By incubation of pH LAL enzyme with cultured mouse macrophages, J-774E cells, hLAL is colocalized with the lysosomal marker, lysosomal associated membrane protein 1 (LAMP-1)³⁹. This suggests an important protective effect of LAL by locally reducing the accumulated CEs and TGs in the foamy macrophages. Second, LAL promotes lysosomal egress of free cholesterol that modulates cellular lipid biosynthesis mediated by the SREBPs. This would lead to increased egress of free cholesterol from lysosomes that would inhibit the endogenous synthesis of cholesterol and fatty acid through the SREBP system.³⁴ Most administered pH LAL localized to macrophages of the liver and spleen where it hydrolyzed CEs and TGs and reduced tissue lipids in *ldlr*^{-/-} mice. Third, LAL cleaves oxLDL and generates 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE that are ligands for PPAR γ .¹³ The ligand activated PPAR γ could have beneficial effects on the reduction of atherosclerotic lesion by its anti-inflammatory activity.⁵¹⁻⁵⁴ LAL activity is required to produce ligands for PPAR γ from oxLDL¹³ that can contribute to in vivo PPAR γ ligand generation. Because synthetic ligands for PPAR γ , eg, rosiglitazone, reduced dietary induced atherosclerotic lesions in male *ldlr*^{-/-} mice.⁵⁴ The natural ligands generated by LAL lipid hydrolysis might be expected to have similar effects on reducing atherosclerotic lesions by activation of PPAR γ and its anti-inflammatory function.⁵⁴ Here, quantitative analyses showed that hLAL administration in group B mice reduced macrophages in the lesions, but also reduced the areas of extension of collagen positive advanced plaques, cholesterol crystal-rich necrotic cores, and the vessel wall dilation (Figure 5). This suggested that LAL also could have anti inflammatory effects. Alternatively, ligand activated PPAR γ could stimulate the gene expression of LXR α , with downstream stimulation of ABCA1 expression.^{55,56} The outcome of this cascade of gene regulation is to promote cholesterol efflux from macrophages in the lesion. Peritoneal macrophages isolated

from *lal*^{-/-} mice have lower cholesterol efflux than those from *lal*^{+/+} mice (data not shown). These data suggest that LAL derived ligands could promote cholesterol efflux from the cells through the ABCA1 transporter. Finally, recent studies have shown the role of free cholesterol in promoting apoptosis in cultured macrophages.^{57,58} It is not clear whether the free cholesterol has same role in lesional macrophages in vivo. The remaining question here is the balance between the production of free cholesterol by LAL that potentially could be toxic and a cause of macrophage cell death and the production of free fatty acid by LAL that could be a ligand for PPAR γ to activate the cholesterol efflux through LXR α and ABCA1 pathway and anti-inflammatory activity of PPAR γ .

Why does supplemental LAL work? Alternatively, at what level of substrate presentation to the lysosome does endogenous LAL activity become insufficient to maintain normal CE and TG flux through this organelle? Studies in atherosclerotic rabbit model indicated that the density of lysosomes was decreased and lysosomal cholesteryl esters was increased in lesional smooth muscle cells and endothelial cells compared with that in normal vessel walls.⁴⁷ These data suggested that LAL activity in lesional cells was/is relatively decreased compared with that in nonlesional vessel walls. Also, LAL activity tends to decrease with age.⁵⁹ Additional factors include the acetylation and oxidation of LDL leading to modified LDL uptake by unregulated scavenger receptors. Because oxLDL can inactivate lysosomal proteases and cause poor degradation of oxLDL in mouse peritoneal macrophages,⁶⁰ one might speculate that oxLDL could potentially inactivate lysosomal lipases as well. Indeed, oxLDL CE are resistant to lysosomal hydrolysis in human macrophage THP-1 cells.²¹ These data suggest that oxLDL could accentuate lysosomal lipid accumulation in foam cells due to direct effects on LAL. As a result, LAL supplementation could compensate for this loss of endogenous LAL activity and reestablish the lysosomal flux of lipids.

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